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Transcriptional activation of collagenase-3 by transforming growth factor-β1 is via MAPK and Smad pathways in human breast cancer cells

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Abstract Transforming growth factor (TGF)-β1, a crucial molecule in metastatic bone cancer, stimulates collagenase-3 expression in the human breast cancer cell line, MDA-MB231. Cycloheximide inhibited this stimulation, indicating that de novo protein synthesis was essential for this response. We examined whether mitogen-activated protein kinase (MAPK) and/or Smad pathways are involved in TGF-β1-stimulated collagenase-3 expression in MDA-MB231 cells. Biochemical blockade of extracellular regulated kinase-1/2 and p38 MAPK pathways partially abolished TGF-β1-stimulated collagenase-3 mRNA expression; whereas overexpression of a dominant negative form of Smad3 completely blocked the TGF-β1-response. These data indicate that TGF-β1-induced MAPK and Smad pathways are involved in TGF-β1-stimulated collagenase-3 expression in MDA-MB231 cells.
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Key words: Extracellular matrix; Collagenase-3; Transforming growth factor-β1 signaling; Breast cancer metastasis

1. Introduction

Matrix metalloproteinases (MMPs) are a family of secreted or transmembrane proteinases that have been implicated in multiple physiological and pathological processes related to extracellular matrix (ECM) turnover, such as normal growth and development, wound healing, angiogenesis, and joint destruction in arthritis [1–4]. MMPs also play an integral role in tumor invasion that is characterized by increased motility of epithelial cells and growth of metastasized tumor cells [5]. Recent studies have shown that collagenase-3 (MMP-13) is overexpressed in a variety of malignant tumors. Human collagenase-3 was originally identified in human breast carcinoma cells [6] and is homologous to rat collagenase-3 [7]. Due to the wide substrate specificity of collagenase-3, the expression of collagenase-3 is very restricted and appears to be subjected to stringent regulatory mechanisms. During tumor growth these controls are lost and tumor cells acquire the ability to produce this protease under stimulation by different factors, including cytokines, growth factors, and tumor promoters. Among them, interleukin (IL) 1α and IL-1β are potential candidates for inducing the expression of this MMP gene in breast carcinomas [8,9].

Transforming growth factor (TGF)-β, a multipotent cytokine enriched in bone matrix, has a wide range of physiological and pathological effects [10–13]. TGF-β1, a crucial molecule in bone metastatic cancer, appears to help guide completion of the bone remodeling cycle. It could also promote breast cancer metastasis by acting directly on the tumor cells [14] via production of parathyroid hormone (PTH)-related protein (PTHrP) that is the critical mediator of bone destruction. The molecular mechanisms responsible for osteolytic metastases are complex and involve bi-directional interactions between tumor cells and bone. One of the possible ways that TGF-β1 is involved in bone destruction is via regulation of MMP activity in metastasizing bone cancer cells. In the present study we show TGF-β1 stimulation of collagenase-3 mRNA expression and secretion in the human breast cancer cell line, MDA-MB231, and the signaling pathways mediating this response.

2. Materials and methods

2.1. Cell culture and growth factor stimulation

MDA-MB231 cells were obtained from ATCC (American Type Culture Collection). The cells were maintained in culture in Dulbecco's modified essential medium (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were treated with TGF-β1 (Invitrogen) or control vehicle in the absence of serum. Inhibitors used in these experiments included the MEK-1 inhibitor PD98059 (Calbiochem), and the p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580 (Calbiochem). Pretreatments with these inhibitors were for 30 min.

2.2. Semiquantitative RT-PCR

Total RNA was prepared using the Qiagen RNeasy kit. RNA obtained from control and TGF-β1-treated MDA-MB231 cells was used for reverse transcription polymerase chain reaction (RT-PCR) by SuperScript One Step RT-PCR kit (Invitrogen). The oligonucleotides (sense 5′-CCTCCTGGGCCAAATTATGGAG-3′ and antisense 5′-CAGCTCCGCATCAACCTGCTG-3′) corresponding to human collagenase-3 were used for specific amplification of a 392 bp fragment of collagenase-3 mRNA. The initial temperature for RT-PCR was 50°C (30 min) and 94°C (2 min) and then 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and elongation (68°C, 1 min). The final extension was at 68°C (7 min). The products were analyzed on a 2% agarose gel.

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Abbreviations: MMP, matrix metalloproteinase; TGF-β1, transforming growth factor-β1; ECM, extracellular matrix; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; RT-PCR, reverse transcription polymerase chain reaction; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase

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2.3. Northern blot analysis

Twenty μg of total RNA per lane was electrophoresed on a 1% agarose, 2.2 M formaldehyde gel in MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate and 1 mM EDTA). RNA was transferred to Zeta-Probe GT-membrane (Bio-Rad) and hybridized in 50% formamide, 5×SSC, 10×Denhardt's, 0.1% SDS, 0.05 M $NaPO_4$ and 100 μg/ml salmon sperm DNA at 42°C. cDNA probes used for hybridization were labeled either with random priming by Prime-a-Gene kit (Promega) or by nick translation kit (Promega). Northern blots were visualized by exposure to film and quantitated by exposure to phosphor screens and analysis in a phosphorimager.

2.4. Quantitative RT-PCR

Total RNA was prepared using the Qiagen RNeasy kit. Reverse transcriptase reaction was carried out using the TaqMan Reverse Transcription reagents (Roche). PCR reactions were performed according to the real-time PCR machine manufacturer's instructions (DNA Engine Opticon, MJ Research, MA, USA), which allow real-time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. The SYBR green kit for PCR reactions was purchased from Perkin Elmer Applied Biosystems. Primers for human collagenase-3 and β-actin were designed using the Primer-Express software (Perkin Elmer Applied Biosystems).

2.5. Western blot analysis

Whole cell lysates from MDA-MB231 cells containing 50 μg of total protein in lysis buffer were electrophoresed by 12% SDS–PAGE. The proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). After blocking in Tween–Tris-buffered saline (0.1% Tween 20, 138 mM NaCl, 5 mM KCl, and 25 mM Tris–HCl, pH 8.0) containing 5% (w/v) non-fat dry milk, the membrane was exposed to primary antibody overnight at 4°C. The membrane was washed and exposed to horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:5000). The antigen–antibody complexes were detected by enhanced chemiluminescence (Amersham Biosciences).

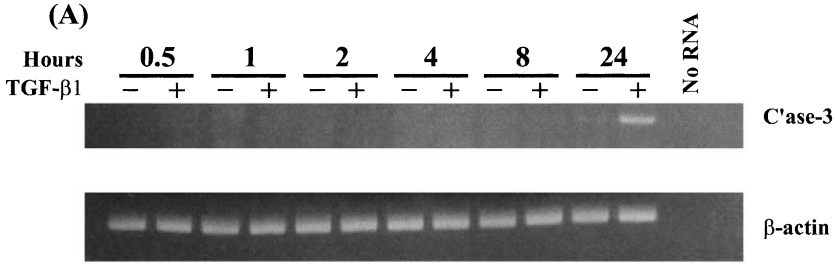
2.6. Transient transfection and chloramphenicol acetyltransferase (CAT) activity

The plasmid DNAs were transiently transfected into breast cancer cells using Lipofectamine (Invitrogen). Briefly, cells were plated at 4×10^5 cells/well in six-well plates in DMEM-F12 containing 10% FBS. The following day, the cells were transfected with 1 μg DNA and 5 μl Lipofectamine per plate in 1 ml of serum-free DMEM-F12. After 16 h, 1 ml of DMEM-F12 containing 10% FBS was added. After 24 h, the cells were treated with either control or TGF-β-containing medium for 24 h. CAT activity was measured by reacting 50 μl of cell lysate in duplicate in a 100 μl reaction volume consisting of final concentrations of 250 μM *n*-butyryl-coenzyme A and 23 mM [^{14}C]chloramphenicol (0.125 μCi/assay). Butylated chloramphenicol was removed by pre-extraction with 200 μl of mixed xylenes. Butylated chloramphenicol retained in the final organic layer was determined by scintillation counting. The values were normalized to protein as determined by the Bradford dye binding (Bio-Rad) method. A standard curve using purified CAT was performed every experiment to determine the linear range of the enzyme assay.

3. Results

3.1. TGF-β1 stimulates collagenase-3 mRNA expression in MDA-MB231 cells and it requires de novo protein synthesis

To study the effect of TGF-β1 on expression of collagenase-



(B)

Treatment	Collagenase-3 secretion (ng/ml)
Control	1.29 ± 0.069
TGF-β1	4.22 ± 0.103[a]

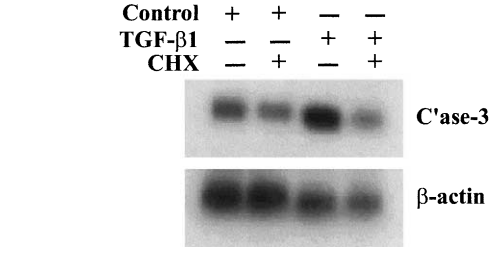


Fig. 1. TGF-β1 stimulates collagenase-3 expression in MDA-MB231 cells and it requires de novo protein synthesis. A: MDA-MB231 cells were serum starved for 24 h and then treated with TGF-β1 (10 ng/ml) at different time periods as indicated. Sense and antisense oligos for human collagenase-3 (C′ase-3) and β-actin were used for RT-PCR. B: MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing TGF-β1 (10 ng/ml) for 24 h. The collagenase-3 levels in the media were measured using an ELISA kit (Amersham Pharmacia). Data represent mean ± S.E.M. of three replicate plates. The statistical analysis was performed using Student's *t*-test and Prism 3.0. [a]Significant difference compared with control ($P<0.001$). C: MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing TGF-β1 (10 ng/ml) for 24 h in the presence or absence of cycloheximide (30 μg/ml, added 1 h before TGF-β1 treatment). Total RNA was subjected to Northern blot analysis and probed with labeled human collagenase-3 or β-actin cDNAs.

3 in human breast cancer, MDA-MB231 cells were treated with TGF-β1 either at different concentrations for 24 h or for different time periods with 10 ng/ml. Total cellular RNAs were purified and analyzed by semi-quantitative RT-PCR. TGF-β1 maximally stimulated collagenase-3 RNA expression at 10 ng/ml concentration in MDA-MB231 cells (data not shown) and requires 24 h for this effect (Fig. 1A). The identity of collagenase-3 (392 bp) and β-actin (661 bp) PCR products was confirmed by transferring PCR products to filters and hybridizing to labeled human collagenase-3 and β-actin cDNA probes, respectively.

An ELISA was then performed using an antibody to human collagenase-3 confirming increased secretion of collagenase-3 into the medium from TGF-β1-treated MDA-MB231 cells (Fig. 1B). To determine if the TGF-β1-mediated increase in collagenase-3 mRNA is a primary response, we used the protein synthesis inhibitor cycloheximide. As shown in Fig. 1C, cycloheximide inhibited TGF-β1 stimulation of collagenase-3 mRNA, indicating that de novo protein synthesis is required for this response.

3.2. Activation of ERK1/2 and p38 MAPKs in MDA-MB231 cells by TGF-β1

Although TGF-β signaling is usually by the Smad pathway in most of the cells studied, some of the biological actions of TGF-β are also mediated by activation of MAPK signaling pathways. To study the role of MAPK (extracellular signal-regulated kinase (ERK) 1/2, p38, and JNK) pathways in the regulation of collagenase-3 expression in MDA-MB231 cells, we first determined the activation of these MAPKs by Western blot analysis of cellular proteins at various time points (0, 15, 60, 120, and 240 min) after exposure to TGF-β1 using antibodies against the active, phosphorylated forms of these MAPKs (Cell Signaling Technology). The levels of activated MAPKs (p-ERK1/2, p-p38, and p-JNK) were quantitated by scanning densitometry and corrected for the levels of total MAPKs (ERK1/2, p38, and JNK) in the same samples. As shown in Fig. 2A, TGF-β1 increased the phosphorylation of ERK1/2 (1.7-fold) at 15 min of stimulation and this reached a maximum at 60 min (2.6-fold). p38 MAPK phosphorylation was increased to a 7.8-fold stimulation at 60 min of TGF-β1 treatment and declined thereafter (Fig. 2B). In contrast to the ERK1/2 and p38 MAPKs, there was no phosphorylation of JNK upon TGF-β1 treatment (Fig. 2C).

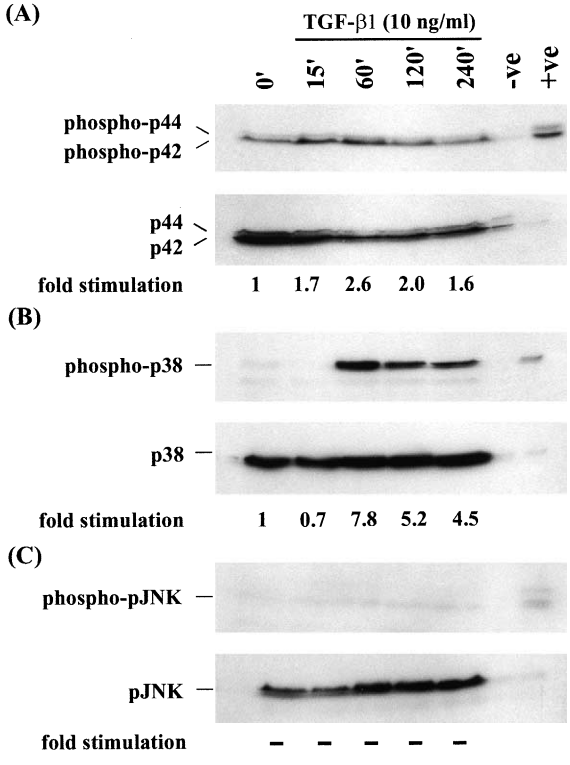


Fig. 2. MAPK pathway activation by TGF-β1. MDA-MB231 cells were incubated with TGF-β1 (10 ng/ml) for the times indicated. A: Phosphorylation of ERK1/2 from whole cell lysates was determined using Western blot with anti-phospho and anti-total ERK1/2 antibodies. B: Phosphorylation of p38 MAPK from whole cell lysates was determined using Western blot with anti-phospho and anti-total p38 antibodies. C: Phosphorylation of JNK MAPK from whole cell lysates was determined using Western blot with anti-phospho and anti-total MAPK antibodies. Cell lysates from C6 cells stimulated with or without anisomycin served as positive (+) and negative (−) controls, respectively. The levels of phospho MAPKs and total MAPKs were quantitated by scanning densitometry and the fold stimulation was calculated over control (0 min).

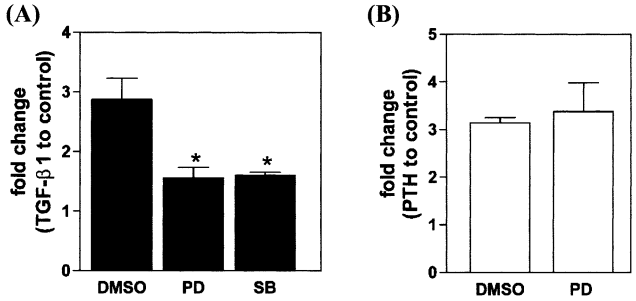


Fig. 3. Inhibition of TGF-β1-stimulated collagenase-3 mRNA expression by ERK1/2 and p38 MAPK inhibition. A: MDA-MB231 cells were pretreated with dimethylsulfoxide (DMSO) or PD98059 or SB203580 for 30 min and incubated in the presence or absence of TGF-β1 (10 ng/ml) for 24 h. Total RNA was isolated and subjected to real-time quantitative RT-PCR using human collagenase-3 and β-actin primers. The relative levels of mRNAs were normalized to β-actin and the change in the levels of collagenase-3 mRNA expression in response to TGF-β1 was calculated as fold change over control. The data are represented as mean ± S.D. ($n = 3$) and were analyzed by ANOVA using Prism 3.0. *Significant difference compared with control (DMSO) ($P < 0.05$). B: UMR 106-01 cells were pretreated with DMSO or PD98059 or SB203580 for 30 min and incubated in the presence or absence of PTH (10^{-8} M) for 24 h. Total RNA was isolated and subjected to real-time quantitative RT-PCR using human collagenase-3 and β-actin primers as mentioned above.

3.3. Inhibition of TGF-β1-stimulated collagenase-3 mRNA expression by ERK1/2 and p38 inhibition

To further elucidate the specific roles of MAPKs in mediating the stimulation of collagenase-3 expression by TGF-β1, we used selective chemical inhibitors of these MAPKs. MDA-MB231 cells were pretreated with PD98059 (25 μM), a specific inhibitor of ERK1/2 kinases MEK1/2, or SB203580 (25 μM), a selective inhibitor of p38 MAPK, for 30 min and then treated with TGF-β1 for 24 h. Total RNA was isolated and subjected to real-time quantitative PCR. Both ERK1/2 and p38 MAPK inhibitors blocked TGF-β1-stimulated collagenase-3 mRNA levels from a fold of 2.876 ± 0.617 to a fold of 1.555 ± 0.306 and 1.603 ± 0.085, respectively (Fig. 3A). In order to show the specificity of MAPK inhibitors, we used rat osteoblastic cells (UMR 106-01) treated with PTH (10^{-8} M).

Both ERK1/2 (Fig. 3B) and p38 MAPK (data not shown) inhibitors had no effect on PTH-induced collagenase-3 expression in UMR 106-01 cells.

3.4. TGF-β1-stimulated collagenase-3 promoter activity depends on the Smad pathway

To determine the functional role of the Smad pathway for TGF-β1-stimulated collagenase-3 expression in MDA-MB231 cells, the rat collagenase-3 promoter containing CAT as a reporter gene [15] was transiently transfected into MDA-MB231 cells along with a dominant-negative form of Smad3 (Smad3M) expression plasmid. The rat collagenase-3 promoter is similar to the human collagenase-3 promoter and most of the regulatory elements present in the rat and human collagenase-3 promoters are highly conserved [15,16]. Overexpression of the Smad mutant significantly abolished both the basal and TGF-β1-stimulated collagenase-3 promoter activity indicating that mutant Smad3 competes with endogenous Smad3 to form a heterodimer with Smad2 and Smad4, thus blocking translocation of the complex into the nucleus (Fig. 4A). In order to determine the specificity of TGF-β

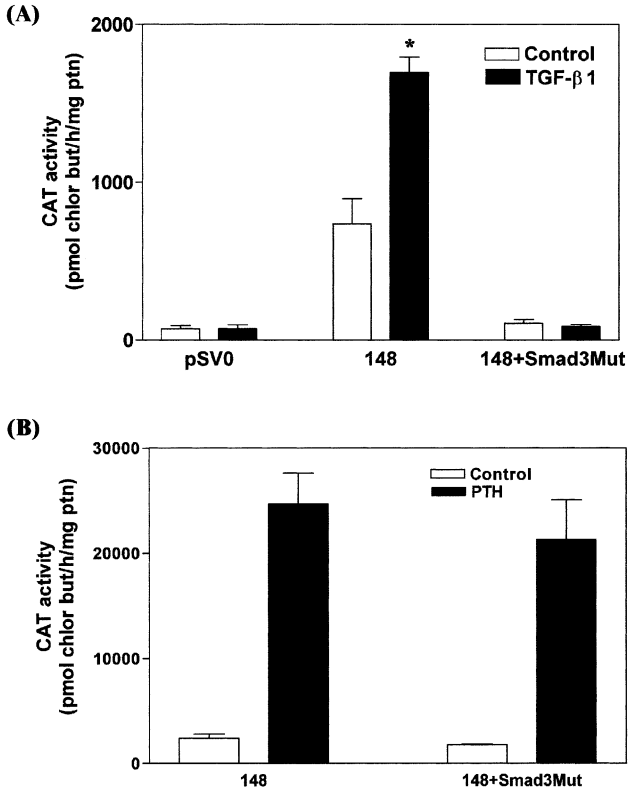


Fig. 4. TGF-β1-stimulated collagenase-3 promoter activation depends on the Smad pathway. A: The wild type collagenase-3 promoter construct (−148) was transiently cotransfected with pCMV-Smad3Mutant (Smad3Mut) construct into MDA-MB231 cells and then treated with control or TGF-β-containing medium for 24 h, and assayed for CAT activity. The total amount of DNA used for all transfections with or without the expression constructs was equalized with pCMV. Data represent mean ± S.D. of three experiments. The data were analyzed by Student's *t*-test and Prism 3.0. *Significant difference compared with control ($P < 0.001$). B: The wild type collagenase-3 promoter construct was transiently cotransfected with pCMV-Smad3Mutant construct into UMR 106-01 cells and then treated with control or PTH (10^{-8} M)-containing medium for 24 h, and assayed for CAT activity. Data represent mean ± S.D. of three experiments.

signaling that is mediated through the Smad pathway for collagenase-3 promoter activity in MDA-MB231 cells, we used the rat osteoblastic cells, UMR 106-01, with PTH (10^{-8} M) treatment. As shown in Fig. 4B, cotransfection of Smad3M did not inhibit PTH-induced collagenase-3 promoter activity in UMR 106-01 cells indicating that Smad-mediated TGF-β signaling in MDA-MB231 cells is specific.

4. Discussion

IL-1α and IL-1β are potential candidates for inducing the expression of collagenase-3 in breast carcinomas [8,9]. TGF-β, a multipotent growth factor highly enriched in bone matrix, induces collagenase-3 expression in human fibroblasts [9]. We here demonstrated that treatment of human breast cancer cells with TGF-β also stimulates collagenase-3 mRNA expression and secretion. Collagenase-3-driven ECM proteolysis may support cancer cell growth both biochemically, by exposing mitogenic factors, and physically, by providing space for the proliferating cells. Since TGF-β-stimulated collagenase-3 expression requires de novo protein synthesis and the collagenase-3 promoter contains an AP-1 site [15], AP-1 transactivation may be required for collagenase-3 expression. AP-1 complex composition can selectively regulate gene transcription and differential expression of Fos and Jun family members could play a role to regulate the expression of downstream target genes [9,17]. We previously showed that differential temporal stimulation of the AP-1 family members may be responsible for collagenase-3 expression in osteoblastic and non-osteoblastic cells [18].

Smad proteins have been shown to mediate the transcriptional activation of various TGF-β-responsive genes such as collagen [19], the tissue plasminogen activator inhibitor [20], the JunB proto-oncogene [21], and the p21/WAF1/Cip1 cell cycle inhibitor [22]. Smad proteins are the main cytoplasmic signaling pathways in TGF-β1-stimulated collagenase-3 expression in osteoarthritic chondrocytes [23]. The Smad proteins are central elements in the TGF-β-receptor signaling pathway but are not the sole pathway activated by this receptor complex. TGF-β family members often require the presence of parallel or synergistic pathways to the Smads to carry out their full biological effects, and diversity of the Smad-interacting partners may contribute to signal specificity [24]. In gingival fibroblasts, TGF-β1 activates both ERK1/2 and p38 MAPK pathways but collagenase-3 expression depends only on the activity of p38 MAPK and the presence of functional AP-1 dimers [25]. The enhancement of collagenase-3 expression by TGF-β1 is also mediated by the p38 MAPK pathway in transformed keratinocytes [26]. In the present study, we evaluated the effect of inhibiting the ERK1/2 or the p38 MAPK pathways on TGF-β-stimulated collagenase-3 mRNA expression in MDA-MB231 cells. Either of them did not completely block the TGF-β-stimulated collagenase-3 mRNA expression indicating the participation of another pathway (Smad) for this effect. Overexpression of the dominant-negative form of Smad3 completely blocked the TGF-β stimulation of the collagenase-3 promoter suggesting that the Smad pathway represents a major pathway for TGF-β in these cells.

The interactions between MAPK and Smad pathways downstream of the TGF-β receptor may be complex. Smad2 and Smad3 are direct substrates for phosphorylation by active

TGF-β type I receptor. Because a dominant-negative form of Smad3 fully suppressed TGF-β responsiveness, activation of the ERK and p38 MAPK pathways would result from tyrosine kinase receptors rather than from serine/threonine kinase receptors (TGF-β receptor). Recently it has been shown that TGF-β-stimulated fibronectin expression is mediated by epidermal growth factor receptor transactivation, and subsequent activation of ERK and p38 MAPKs [27]. There may be cross-talk between the MAPK and Smad pathways for TGF-β-stimulated collagenase-3 expression in MDA-MB231 cells. For example, MAPKs phosphorylate Smad2/3 proteins other than the SSXS motif activated by the TGF-β type I receptor. Cross-talk between Smad, ERK1/2, and p38 MAPK pathways for TGF-β induction of the aggrecan gene has been reported [28]. TGF-β1 stimulation of PTHrP is also dependent on Smad and MAPK pathways [29]. Overall, our studies demonstrate transcriptional activation of the collagenase-3 gene by TGF-β1 in human breast cancer cells and that transcriptional activation is mediated by both the MAPK and Smad pathways.

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